The Role of Human Papillomavirus 16 E6 in Anchorage-Independent and Invasive Growth of Mouse Tonsil Epithelium

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Objective: To provide a manipulatable system to study the mechanism of human papillomavirus 16 (HPV16) E6-related transformation of an epithelial cell type affected by HPV16 in humans.

Design: Biochemical and physiological studies of mouse tonsil epithelial cells (MTECs) transformed with HPV16 oncogenes plus H-ras in vitro and in vivo.

Setting: Basic research laboratory.

Participants: C57BL/6 mice.

Interventions: Transduction of the HPV16 oncogenes E6 and E7 in retroviral vectors into MTECs with isolation of multiple individual clones that expressed E6, E7, or both alone or in conjunction with H-ras.

Main Outcome Measures: Growth in culture, anchorage-independent growth, and growth in immune competent, syngeneic mice.

Results: The MTECs that expressed E6 degraded p53 by a mechanism that is inhibited by proteasomal blockade. Although normal MTECs senesced after 20 population doublings, E6 alone or in combination with E7 was sufficient to immortalize MTECs beyond 25 population doublings, lower their population-doubling time, and permit anchorage-independent growth. However, only MTECs that express E6 plus H-ras or E6/E7 plus H-ras formed invasive tumors in immune competent, syngeneic mice at orthotopic intraoral and subdermal sites.

Conclusions: We found that HPV16 E6 and E7 alone are not sufficient for invasive growth. However, the synergistic activity of H-ras and E6 was sufficient to result in invasive growth.

Arch Otolaryngol Head Neck Surg. 2007;133:495-502

The incidence of head and neck squamous cell carcinoma (HNSCCA) has increased worldwide.¹ Despite advances in surgical and combined chemotherapy and radiation therapy, the overall survival of patients with HNSCCA has not improved during the last 30 years.² Staging of HNSCCA is based on local invasion and nodal or distant spread. Current treatment of early-stage (stage 1 and 2) HNSCCA produces good 5-year disease-specific survival rates (81%), whereas late-stage tumors have poor survival rates (50%).³ A significant reason for the unchanged survival rates is a lack of effective treatments for late-stage cancers that exhibit aggressive local invasion or distant metastasis at the time of presentation.² The mechanisms that control metastasis and aggressive invasion are not understood. To design better therapies to improve survival, further investigation into mechanisms of malignant transformation are essential.

One of the obstacles in developing treatments for metastatic disease will be determining the particular pathways that transform a cell and result in metastatic growth. Once a mechanism is defined, it would be possible to develop a treatment based on a specific cellular alteration. For any individual cancer it is likely that many different combinations of cellular pathways could cause a malignant phenotype; therefore, a successful mechanism-based treatment would be needed to identify susceptible tumors with a particular altered cellular pathway before treatment. Treatment of chronic myelogenous leukemia containing the bcr-abl translocation with imatinib mesylate is a successful example of such a strategy.⁴ For many HNSCCAs, pretreatment identification of a malignant mechanism is currently not possible; however, human papillomavirus (HPV)-related HNSCCA represents a subset of cancers in which
plays in malignant transformation has predominantly been tonsil epithelial keratinocytes. Currently, the role HPV16 transformation will require in vitro and in vivo studies in the next several decades.

The study of HPV has taught us a great deal about how human cells become transformed. For example, the demonstration that the E6 and E7 genes from high-risk HPV types can immortalize human cells along with the finding that E6 and E7 proteins inactivate the p53 and pRb pathways gave significant insight into the involvement of these pathways in carcinogenesis. Recent advances in the development of prophylactic vaccines for HPV infection were a direct result of studies on HPV biological mechanism. However, the hundreds of millions of people infection were a direct result of studies on HPV biological mechanism. Understanding HPV-related mechanisms of malignant transformation will require in vitro and in vivo studies in tonsil epithelial keratinocytes. Currently, the role HPV16 plays in malignant transformation has predominately been investigated in anogenital keratinocytes, and some of the mechanisms of transformation are known. Although some of these mechanisms regarding transformation of anogenital keratinocytes will likely also relate to tonsillar keratinocytes, there will be differences. Therefore, to best understand the mechanisms of HPV-related transformation in HNSCCA, it is necessary to complete studies in keratinocytes from the most affected area (tonsil).

In addition to studying transformation in tonsil cells in vitro, a mechanistic-based therapy will also be aided by an in vivo model. Such a model would not only allow for the exploration of HPV-related mechanisms responsible for in vivo invasion and metastasis but would also be a valuable preclinical testing site for future therapies. Many different strategies can be pursued to develop such a model. Although it may be possible to test xenografted human tonsil cells with viral genes in immunoincompetent mice, this model has significant limitations (e.g., aberrant immune response and species differences in receptors and ligands related to metastatic growth). The lack of an immune response in this model is a significant problem because several lines of evidence suggest that HPV subverts the immune response to its advantage to encourage growth. A transgenic mouse model of HPV16-related skin cancer exists. In this transgenic mouse model, the human cytokeratin 14 (K14) promoter drives expression of HPV16 E6 and E7. The HPV mice develop squamous cancers of the skin (14% in 2 years). We present a new nontransgenic approach to study HPV-related tonsil cancer for several reasons: (1) the current transgenic model does not develop tonsil cell cancer, (2) the development of transgenic mice requires substantial cost and prolonged time to observe results with a low frequency of tumor formation, (3) multiple genetic changes in a mouse can be difficult to study because of embryonic lethality, and (4) the immune reaction to HPV antigens may aid the development of HPV-related malignancy. In the current transgenic mouse model, the mice would be tolerant to viral antigens because they are present throughout development. In our approach, we can rapidly test many hypotheses in mice with many genetic alterations (e.g., knockout mice, mice with conditional expression of genes, or immune system variants). Our model also exposes the mice to HPV genes only after implantation in a synergistic immune competent animal. This method will allow us to understand invasive neoplastic growth in the context of an immune competent animal.

A limitation of using mice to study human disease is that species differences are present. A mouse model that accurately reflects human disease would provide an adequate testing ground for studies designed to discover basic mechanisms of carcinogenesis and for testing preclinical therapies. We describe a method to culture mouse tonsil epithelial cells (MTECs). We deliver HPV oncogenes to MTECs to determine what viral genes are required for immortalization, anchorage-independent growth, and in vivo malignant growth. To begin to determine the human relevance of studying HPV transformation in MTECs, we also examine whether the viral HPV oncogenes cause similar cellular changes in mouse and human cells.

METHODS

ISOLATION AND ESTABLISHMENT OF MTECs

The C57BL/6 mice were euthanized, and the oral epithelium overlying the tonsillar fossa was harvested. Institutional guidelines regarding animal experimentation were followed. The epithelium was dissociated from the underlying dermis with overnight Dispase II treatment (0.25 g/10 mL; Roche Applied Science, Indianapolis, Ind) manual tissue separation, brief trypsinization with 0.25% trypsin (Invitrogen Corporation, Carlsbad, Calif), and subsequent outgrowth in keratinoocyte serum-free media (Invitrogen) that contained 0.2-ng/mL epidermal growth factor, 25-µg/mL bovine pituitary extract (Invitrogen), 1% penicillin/streptomycin, and 25-µg/mL amphotericin B (Fungizone; Gibco Invitrogen Corp, Carlsbad, Calif). After the first passages, the cells were grown in Dulbecco's modified Eagle medium that contained 10% fetal bovine serum, 22% Ham's F12, 1% penicillin/streptomycin, 25-µg/mL hydrocortisone, 8.4 ng/mL cholera toxin, 5-µg/mL transferrin, 5-µg/mL insulin, 1.36-ng/mL triiodothyronine, and 5-ng/mL epithelial growth factor. Cells in serum that contained media required the addition of irradiated murine J2-3T3 feeder cells for maximal growth and viability.

RETROVIRAL PRODUCTION, TRANSDUCTION, AND GENERATION OF STABLE LINES

Replication incompetent retrovirus was produced by plasmid transfection in packaging cell lines using previously reported techniques. Early passage MTECs were transduced at passage 2. Retroviruses that expressed E6, E7, H-ras, and a control empty vector (LXSN) were used to transduce approximately 40% confluent normal mouse tonsillar cells for 12 hours at 37°C in 5% carbon dioxide. After media change, cells were cultured for 16 hours, split in a 1:4 ratio, and subjected to initial antibiotic selection. Transduced cells were selected by the addition of 150-µg/mL G418 (Invitrogen Corporation) for E6 and LXSN, 150-µg/mL hygromycin (Invitrogen) for E7, and
1-µg/mL puromycin for H-ras beginning 24 hours after retroviral transduction. Cells were maintained at this high antibiotic concentration until 100% of the untransduced control cells had died (approximately 3 to 10 days). Stable expressing cell lines were maintained in 50-µg/mL G418, 1-µg/mL hygromycin, and 0.2-µg/mL puromycin, respectively. During in vitro culture the cells were split in a 1:4 ratio, which represents 2 population-doubling events.

Clonal cell lines from these pooled transductions were generated by splitting pooled populations in a 1:100 or 1:1000 ratio onto 10-cm tissue culture plates and isolating single-colony clones. These clonal populations were ring cloned using 8-mm rings (Fisher Scientific Company LLC, Pittsburgh, Pa). More than 6 clones were initially isolated from each transduction condition (E6, E6 and E7, and H-ras). In experiments with LXSN and E7 transduced cells, assays were performed on pooled populations because the cells underwent senescence before cloning could be completed. Each transduction condition was repeated multiple times.

**CELL LYSIS AND IMMUNOBLOT**

Whole cell lysates were harvested at 4°C with protein lysis buffer that consisted of 30mM Tris hydrochloride (pH, 7.5), 150mM sodium chloride, 5mM EDTA, 2mM sodium orthovanadate (Na3VO4), 10mM sodium inorganic pyrophosphate (NaPP), 100mM sodium fluoride (NaF), 10% glycerol, 1% Triton X-100, 10-µg/mL pepstatin, 20-µg/mL leupeptin, and 20-µg/mL aprotonin. Lysates were drawn through a 23-gauge needle 10 times to ensure complete protein dissociation from other cellular components. Cell pellets were snap frozen at −80°C until use. Expression of p53 and actin was measured by immunoblotting with a standard Western blot technique. Blots were developed using the Super Signal West Pico Kit (Pierce Biotechnology, Rockford, Ill).

**MG132 INHIBITOR ASSAY**

The MTECs transduced with LXSN or HPV16 E6 and E7 were grown in culture at 70% confluency then treated with 50µM proteasomal inhibitor MG132 (2211, Sigma-Aldrich Corp, St Louis, Mo) for 3 hours. Controls received only a media change. Total cell lysate was harvested for protein, and a Western blot analysis for p53 and actin was performed as described herein.

**ANCHORAGE-INDEPENDENT GROWTH**

Cells were grown to 70% confluency, then harvested with trypsin. A 0.66% noble agar was seeded onto 12-well plates and allowed to congeal. A mixture of 0.33% noble agar and 1 × 10^6 cells was applied onto the 0.66% agar and set for 15 minutes at 4°C. Cells were grown at 37°C in 5% carbon dioxide for 10 days with feeding of 0.33% agar every 2 days. Colonies greater than 50 cells were counted in quadrants on day 10 with triplicate wells for each cell line.

**IN VIVO TUMOR GROWTH**

Cells were harvested at 70% confluence with trypsin and re-suspended in normal growth media with a concentration of 1 × 10^6 cells/mL. Using an 18-gauge needle, C37BL/6 mice were injected with either 5 × 10^6 cells in the tongue or 1 × 10^6 cells in the subcutaneous tissue in the upper dorsal quadrant near the spine using halothane anesthesia. Animals were killed manually when tumor size was larger than 20 mm in its greatest dimension, the animal was substantially emaciated, or pain or functional impairment (jaw or leg use) was apparent. Tissue was preserved in formalin (2%) and sectioned for hematoxylin-eosin staining per standard protocol. Histologic findings were analyzed by a pathology specialist in head and neck cancer.

**RESULTS**

**ESTABLISH A METHOD TO ISOLATE AND CULTURE MTECs**

The highest percentage of HPV-positive HNSCCAs originates from tonsillar epithelial cells. To establish a relevant cell culture model, we developed a method to isolate and culture tonsil epithelial cells from the mouse tonsillar fossa. After the first passage in serum-free media to eliminate contamination with fibroblasts, the cells were plated with irradiated feeders and the media was switched to a basal media with serum. Under these conditions, the cells grow in epithelial colonies (Figure 1). The cells in these conditions can be propagated for up to approximately 15 to 20 population doublings. However, in rare instances clonal populations of morphologically normal MTECs can be cultured for up to 30 to 40 population doublings before undergoing cell death. This extremely prolonged life of rare clones has also been described for other mouse cells in culture. Therefore, in the subsequent studies that determined immortality related to a particular viral oncogene, we found it necessary to not only follow pooled populations of transduced cells but also to quantify the number of clones that obtained immortality after a retroviral transduction. In subsequent experiments with retroviral insertions of HPV16 oncogenes, MTECs were not considered immortal until they had exceeded 80 population doublings. We did not consider an event related to a transgene unless it occurred in more than 90% of the clones isolated after a transduction or repeated on multiple pooled transductions.

**E6 ALONE RESULTS IN MTEC IMMORTALIZATION AND ALTERED MORPHOLOGIC FINDINGS**

In culture, epithelial cells have a limited lifespan with eventual apoptosis, cell cycle arrest, or senescent cell death. Expression of E6 and E7 oncogenes provides mechanisms that result in efficient immortalization of human tonsil cells (J.H.L., unpublished data, 2004) and other human keratinocytes. The requirements for immortalization of MTECs have not been previously explored. However, other mouse cells apparently require fewer altered cellular pathways to result in immortalization. We used retroviral transduction of HPV16 oncogenes into MTECs in culture to determine what viral oncogenes are required for immortalization.

HPV16 E6 alone was sufficient to alter morphologic findings and immortalize MTECs. Immediately after selection, it became apparent that clones that contained E6 alone and E6 and E7 combined grew in small, tight colonies (Figure 1). The cells continued to grow as epithelial colonies, displacing the irradiated fibroblasts. In con-
Contrast, primary mouse tonsil cells transduced with the empty vector LXSN formed colonies with large senescent cells at the center (Figure 1). We continued to passage these cells to determine which genes were required for immortalization in vitro. Expression of E6 by itself resulted in the immortality of MTECs (Figure 2), whereas control cells (LXSN) and cells that expressed E7 alone did not immortalize. Compared with E6 alone, clones that expressed both E6 and E7 showed a slightly faster overall growth rate in vitro. Thus, unlike human tonsil cells (J.H.L., unpublished data, 2004), mouse tonsil cells are immortalized with E6 alone with no added requirement of the degradation of pRb by E7.

**E6 DEGRADATES MOUSE p53 VIA PROTEASOMAL-MEDIATED DEGRADATION**

HPV16 E6 has been shown to decrease apoptosis in anogenital keratinocytes by proteasomal-mediated degradation of p53.\(^\text{10}\) We investigated whether human viral protein E6 would inactivate mouse p53 by similar mechanisms. Indirect evidence showing lack of p53 induction in the K14-16E6 mouse after irradiation suggested that E6 degrades p53 in the mouse.\(^\text{11}\) After retroviral transduction, we assayed for p53 degradation using Western blot analysis. The results (Figure 3) show that in the presence of E6, mouse p53 is almost undetectable. In addition, p53 rescue with proteasome inhibition suggests that targeted degradation occurs by a similar pathway in mouse and human tonsil cells.
cells (Figure 4). These findings suggest that the mouse may be a suitable model to study the mechanisms of E6-mediated transformation.

INDUCTION OF ANCHORAGE-INDEPENDENT GROWTH BUT NOT TUMOR GROWTH IN VIVO BY E6

In the progression to neoplasia, an immortal cell must acquire the ability to grow in the absence of regulatory message from the extracellular environment, such as the basement membrane. To determine the transformed state of the HPV-immortalized MTECs, we tested their ability to induce anchorage-independent growth in vitro or form tumors in immune competent, syngeneic mice. Anchorage-independent growth in many circumstances predicts tumor growth in vivo.20 As shown in Figure 5, E6-transformed cells were able to grow in an anchorage-independent manner. The addition of E7 to E6 increased the efficiency of anchorage-independent growth in vitro. Although anchorage-independent growth was observed for the E6-transformed cells and the E6 and E7 cells, tumor growth in vivo was not seen (Table). These data suggest that HPV viral oncogenes are sufficient to induce anchorage-independent growth of MTECs in vitro but alone are not sufficient for in vivo tumor formation.

H-ras SYNERGIZES WITH E6 TO INDUCE TUMOR GROWTH IN VIVO

In our HPV-transformed cells, we chose to express an additional active-form oncogene (H-ras) and test for in vivo growth for several reasons: (1) ras is overexpressed and is implicated in HNSCCA.21,22 and (2) it has been shown to synergize with E6 and E7 tumor formation in mice.23,24 We transduced H-ras or a control retroviral vector into E6 alone and E6 and E7 combined MTECs. Pure cultures of transduced cells were obtained through selection with puromycin. H-ras transduction of these cells greatly increased their ability to grow in an anchorage-independent manner; however, anchorage-independent growth did not occur in the presence of H-ras alone (Figure 5). These cells were also transferred either under the epithelial lining of the mouth or subcutaneously in the upper dorsal quadrant near the spine. Aggressive, invading tumors developed at both sites within 1 week (Figure 6). Histologic analysis of the tumors showed that they are poorly differentiated squamous cell cancer (Figure 6). Aggressive local invasion of muscle and fat, as well as capillary lymphatic space invasion, was present in all specimens. Vector control cells and cells transduced with H-ras alone did not grow in mice (Table). Although overall survival was similar, survival time was substantially decreased in mice with orthotopic oral, as opposed to flank, tumors (Figure 7A). This finding likely represents the associated effects of local tumor volume on oral intake. Evaluation of tumor growth using either direct caliper measurement or indirect luciferase-mediated light expression shows logarithmic growth (Figure 7C). The cellular changes associated with individual expression of H-ras, E6, or E6 and E7 combined are not sufficient for invasive growth; however, the complement of the cellular

[Figure 3. Selective degradation of p53 by human papillomavirus 16 E6. Mouse tonsil epithelial cells transduced with LXSN, E6, or E6 and E7 combined were analyzed by Western blot probed with p53 and actin (loading control). Two representative clones of E6 and E6 and E7 combined are shown (A and B). The 50- and 37-kDa molecular weight markers are shown.]

[Figure 4. Proteasomal dependence of E6-related p53 degradation. Mouse tonsil epithelial cells transduced with LXSN or E6 and E7 were treated with 50µM proteasomal inhibitor MG132 (2211; Sigma-Aldrich Corp, St Louis, Mo) for 3 hours. The Western blot was probed with antibodies to p53 and actin (loading control).]

Human papillomavirus plays a critical role in malignant transformation of tonsil epithelial cells. Our data show that we have the ability to alter tonsil cells in culture using viral oncogenes and correlate phenotypic changes in their growth both in vitro and in vivo with a syngeneic, immune competent mouse. Previous studies in other mouse cell types not associated with HPV-related disease have used HPV viral oncogenes and ras to obtain invasive growth.25-28 However, our data expand on these previous studies in the following ways: (1) the use of an epithelial cell type harvested from a common site of HPV-related cancers, (2) individual examination of which viral
oncogenes are required for in vivo invasive growth, (3) use of a quantitative detection mechanism to monitor neoplastic growth, and (4) examination of multiple in vitro characteristics associated with transformation (anchorage-independent growth, immortality, and growth rates).

For a mouse model to be helpful in understanding human disease, the cellular mechanisms and disease process should be similar. Our findings show that in mice, E6 expression results in loss of p53. Furthermore, we show that this E6-induced loss is likely mediated by proteasomal degradation because it is reversible with MG132 (proteasome inhibitor). These findings are consistent with the E6-mediated loss of p53 in the human epithelium. Therefore, it would appear that the mouse could be an excellent model to study the role of E6 in tonsil cell transformation. Although our findings on E6 function in mouse cells show similarity in the mechanism of p53 degradation, E6 may have additional functions in mouse cells not present in a human cell.

Our data also suggest that mice are different from humans in the HPV genes required for immortalization. Human tonsil cells and anogenital keratinocytes require the expression of both E6 and E7 for immortalization.

Therefore, we were initially surprised to find that E6 alone results in immortalization of mouse tonsil cells. Other studies that examined the requirements for mouse cell immortalization have found that pRb inactivation is not required. Transgenic E6 mice develop tumors in the absence of E7 expression. It is possible that E7-mediated pRb inactivation still occurs in the cells with both E6 and E7. We noticed a slight increase in growth rate and increased anchorage-independent growth in the cells with both E6 and E7 compared with E6 alone. However, preliminary studies in our laboratory have shown that, unlike human cells, E7 does not induce p16 expression in MTECs (data not shown). It is possible that the viral genes mechanistically alter mouse cells differently than they do human cells. Future studies are currently planned to correlate other known interactions of HPV16 E6 and E7 with cellular targets in human cells with mechanistically similar functions in mouse cells.

This study determines the requirements of transformation of mouse tonsil cells in the context of the major transforming oncogenes of HPV16 (E6 and E7). We did not investigate the effects, possibly synergistic or antagonistic, of other HPV16 genes. Interestingly, other known early HPV genes may also play a role in transformation. For instance, E5 has been shown to alter epidermal growth factor receptor processing. The absence of all the HPV16 genes could limit the interpretation of investigations of immunity or tumor clearance. Using our methods, it would be possible in future studies to systematically examine the addition of other viral genes with regard to transformation and invasive growth.

Our findings also suggest that HPV-related carcinogenesis is a multistep process. We have found that expression of HPV viral oncogenes alone is not sufficient to permit metastatic growth. This result supports the clinical finding that not all patients with persistent HPV infection develop cancer. It is likely that at least 1 additional cellular mutation in the presence of HPV oncogenes is required for metastatic growth. The E6 transgenic mouse data support these findings. In one study, only 14% of mice that overexpressed E6 developed malignant tumors, suggesting that E6 is not sufficient to induce tumor growth by itself. Chromosomal analysis of the tumors that developed in these mice indicated that they exhibited other genetic alterations compared with the sur-

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**Table. Summary Population Data**

<table>
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<th>Retrovirus</th>
<th>PD Time, d</th>
<th>Immortal</th>
<th>Clones Immortal, No./Total No.</th>
<th>Anchorage-Independent Growth</th>
<th>Growth In Vivo, No./Total No.</th>
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<td>−</td>
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<td>0/10</td>
<td>−</td>
<td>NT</td>
</tr>
<tr>
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<td>0/10</td>
<td>−</td>
<td>NT</td>
</tr>
<tr>
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<td>+</td>
<td>10/10</td>
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</tr>
<tr>
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<td>50</td>
<td>−</td>
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<td>NT</td>
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<tr>
<td>E6 plus E7</td>
<td>12</td>
<td>+</td>
<td>10/10</td>
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<tr>
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<td>10/12</td>
<td>+</td>
<td>0/6</td>
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Abbreviations: NA, not applicable; NT, not tested; PD, population doubling; −, negative; +, positive.

*Mouse tonsil epithelial cells transduced with retroviruses that contained oncogenes with clonal populations obtained in lines that survived subcloning.

†Cells did not survive selection.

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**Figure 5.** Colony-forming efficiency (CFE) in agar. Transduced mouse tonsil epithelial cells (MTECs) were suspended in semisolid agar. Colonies greater than 1 mm were counted under ×4 magnification on day 10 after seeding. The experiment was performed in triplicate and repeated. Error bars indicate standard deviation.
rounding tissue. The fact that H-ras synergizes with E6 to result in invasive growth may shed light into what other cellular pathways need to be altered to result in invasive tumorigenic growth. H-ras activation is a downstream event of growth factor signaling. Growth factor receptor overexpression is common in HNSCCA. Although H-ras mutations have not been reported in HPV-related HNSCCA, it is possible that alterations such as growth factor receptor overexpression or other components of the growth factor signaling cascade could synergize with HPV oncogenes to allow invasive growth.

Other than testing what mechanisms are critical for HPV-related carcinogenesis, this model has several other practical applications and advantages. From a cost and time perspective, an in vivo approach takes months to test a hypothesis and is relatively cost-effective compared with completing the same study by developing a transgenic or knockout animal. It is also possible to test cellular alterations using this approach that are not possible using a genetic approach. Cellular changes that would cause embryonic lethality in a transgenic mouse could be introduced into the mature mouse. This approach also mimics cancer development because the cancer is introduced into an immune competent animal. With the current HPV transgenic mice, the mice become tolerant to the viral proteins because the genes are present throughout development. Therefore, in our model, a mouse may be induced to immunologically clear the tumor by strategies that target the viral antigens, although a limitation is the presence of only 2 HPV16 oncogenes out of the full HPV16 genome. We chose retroviral transduction to insert oncogenes because it offers stable expression and selection over time. Also, multiple retroviruses that contain various genes can be made inexpensively with little time and resources compared with adenovirus production. Transduction of oncogenes using retroviruses can lead to suppression of cellular genes owing to insertional events; however, multiple clonal cell lines that show similar results and the use of empty vector controls confirm that the effects seen are due to the oncogenes and not random insertions. These methods provide a means to reproducibly observe gene-induced changes in cells that will best be confirmed by validations of human tumor specimens.

In conclusion, these data present a method to study the HPV viral genes required for specific aspects of transformation in primary mouse tonsil keratinocytes. Although the HPV viral gene requirements for transformation are different between mouse and human tonsil keratinocytes, the E6-dependent loss of p53 appears similar.
lar. This system may provide a relevant preclinical model for testing potential therapeutic strategies.

Submitted for Publication: August 7, 2006; final revision received November 15; accepted January 9, 2007.

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Author Contributions: Drs Spanos, Harris, and Lee had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Spanos, Harris, Klingelhutz, and Lee. Acquisition of data: Spanos and Anderson. Analysis and interpretation of data: Hoover, Spanos, Klingelhutz, and Lee. Drafting of the manuscript: Spanos, Harris, Anderson, Klingelhutz, and Lee. Critical revision of the manuscript for important intellectual content: Hoover, Spanos, Klingelhutz, and Lee. Obtained funding: Klingelhutz and Lee. Administrative, technical, and material support: Hoover, Spanos, Anderson, Klingelhutz, and Lee. Study supervision: Harris, Klingelhutz, and Lee. Drs Hoover and Spanos contributed equally to this work.

Financial Disclosure: None reported.

Funding Support: This study was funded by Veterans Administration Medical Center and National Institute of Health grant K08.

Previous Presentation: This study was presented at the American Head and Neck Society 2006 Annual Meeting and Research Workshop on the Biology, Prevention, and Treatment of Head and Neck Cancer; August 18, 2006; Chicago, Ill.

Additional Information: Mr Hoover is a Howard Hughes Medical Institute Medical Research Training Fellow. Acknowledgment: We thank Andrea Van Gelder for manuscript preparation.

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